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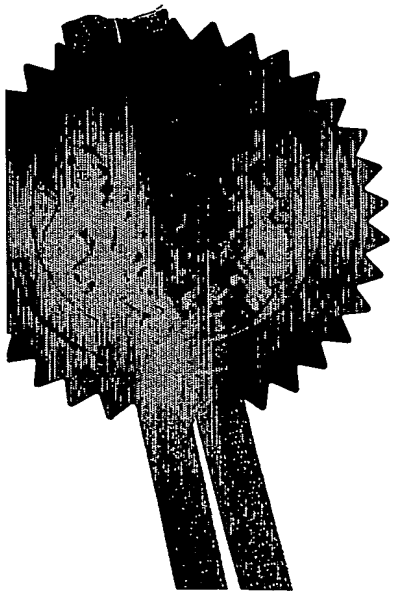

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Dated 25 May 2004

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22 APR 2003

0309064.4

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

The Victoria University of Manchester
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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

UK

4. Title of the invention

MODIFIED PEPTIDES AND THEIR USES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

ROYSTONS

Tower Building,
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Patents ADP number *(if you know it)*

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Claim *(s)*	-
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Title: MODIFIED PEPTIDES AND THEIR USES

DESCRIPTION

The present invention relates to modified extracellular matrix molecules, to polymers, matrices and gels made therefrom and to their uses in such applications as wound healing.

There is a need for new clinical therapies to treat chronic wounds. The wound care market is vast and the cost to health authorities treating foot and leg ulcers is an estimated $7,000 million p.a. worldwide (FDA website http://www.fda.gov/). The existing treatments for such wounds include glutaraldehyde –cross-linked collagen implants, type I collagen gels containing cultured fibroblasts or fibroblasts supported on polyacid substrates. The use of chemical substrates, exogenous cells and crosslinking compounds increases the risk of implant rejection, antigenic responses and poor integration at the wound margin. Also, dressings containing pre-cultured cells are difficult to scale up and deliver fresh to the patient.

Furthermore, the standard treatment for chronic wounds, such as venous ulcers, is the use of absorbent or non-absorbent dressings in conjunction with compression therapy. However, this approach is only moderately effective, is uncomfortable for the patient, can take several months to take effect and recurrence occurs in the majority of cases where treatment is completed. Therefore, there is a an urgent need for the treatment and management of chronic wounds that avoids repeated applications of expensive dressings and which fail to address the underlying cellular and molecular mechanisms contributing to the pathogenesis of delayed healing. One

of the most important contributing factors that results in the standard treatments for wound healing being only moderately effective is the markedly reduced deposition of collagen at the wound site associated with impaired cellular infiltration.

Most cells, whether simple unicellular organisms or cells from human tissue, are surrounded by an intricate network of macromolecules which is known as the extracellular matrix (ECM) and which is comprised of a variety of proteins and polysaccharides. A major protein component of the ECM is a family of related proteins called the collagens which are thought to constitute approximately 25% of total proteins in mammals. There are at least 26 genetically distinct types of collagen molecule, some of which are known as fibrillar collagens (collagen types I, II, III, V and XI) because they typically form large fibres, known as collagen fibrils, that may be many micrometers long and may be visualised by electron microscopy.

Collagen fibrils are comprised of polymers of collagen molecules and are produced by a process involving conversion of procollagen to collagen molecules that then assemble to form the polymer. Procollagen consists of a triple stranded helical domain in the centre of the molecule and has non-helical domains at the amino terminal (known as the N-terminal propeptide) and at the carboxyl terminal (known as the C-terminal propeptide). The triple stranded helical domain is made up of three polypeptides which are known as α chains. Procollagen is made intracellularly from pro-α chains (α chains with N and C-terminal forming propeptides domains). Pro-α chains are synthesised on membrane-bound ribosomes following which the pro-α chains are inserted into the endoplasmic reticulum. Within the endoplasmic reticulum the pro-α chains are assembled into a procollagen molecule. Procollagen is secreted into the extracellular environment where it is then converted into collagen by the action of procollagen N-proteinases (which cleave the N-terminal propeptide) and procollagen C-proteinases (which cleave the C-terminal propeptide). Once the propeptides have been removed the collagen molecules thus formed are able to self-

assemble spontaneously to form the collagen fibrils. The rate determining step in the formation of collagen fibrils is the removal of the C-propeptides by procollagen C-proteinases.

Collagen fibrils interact with other fibrils and also other components of the extracellular matrix to form connective tissues *in vivo*. Fibrils will assemble *in vitro* and will interact to form a collagen matrix or gel. Such collagen matrices have various industrial uses. For instance, collagen-based biomedical products are used in the cosmetic and aesthetic enhancement markets as implants and for smoothing lines, wrinkles and facial scars. Collagen based products are also used in the production of artificial skins (e.g. for treating burns patients), wound dressings and the like.

Whilst collagen based products have been extensively adopted, their performance is far from satisfactory and a number of contra-indications and adverse reactions are known. Some of the problems are related to the fact that many of these products are based on animal collagen (e.g. from bovine hide) and as such can give rise to allergic and inflammatory reactions and infections. Other collagen products are derived from cadaver tissue and it is suggested that they result in reduced inflammation and allergic reactions. However such products are expensive to manufacture and difficulties in controlling product quality can lead to variation in performance.

Another important function of the ECM is the storage and presentation of growth factors to cells. Proteoglycan components of the ECM play a central role in the regulation of the activity of a number of growth factors and therefore represent powerful pathophysiological modulators.

A well known example of a family of proteoglycans has a core protein of about 40 kDa that consists mainly of leucine-rich repeats of 20 - 24 amino acids. These proteins are known as Small Leucine-Rich Proteoglycans (SLRPs) and typically contain the sequence $LX_3LXLX_2NX(L/I)$ where L = leucine; N= asparagine are in the specified conserved positions and X = any amino acid.

The SLRP family comprises at least 4 members, namely decorin, biglycan, fibromodulin and lumican (all of which were characterised in some detail in the late 1980s/early 1990s). These proteoglycans have specialised functions in cell cycle regulation, in tissue repair and in modulating the mechanical properties of tissues by their interaction with collagen fibrils. Decorin and related proteoglycans have also been reported to bind to and modulate the activity of various growth factors including members of the transforming growth factor β (TGF-β) family. Growth factors such as the TGF-βs have a major influence on cell activity and ECM remodelling. There are at least 5 different TGF-βs (TGF-β1 - TGF-β5) and their chemical structures and activity have been widely reported (e.g. see Sporn *et al.* J. Cell Biol. 105: 1039 (1987).

A major pathophysiological activity of TGF-βs (particularly TGF-β1 and TGF-β2) is the promotion of wound healing. However this is often associated with increased scar formation and fibrosis. In fact, clinical interest in the modulation of TGF-β has been associated with inhibiting its activity in order to reduce scar formation (although this may compromise the rate of wound healing). For instance, WO 92/17206 discloses compositions which inhibit the activity of TGF-β1 and TGF-β2 and are particularly beneficial for reducing scar formation.

Another proteoglycan that is known to bind to TGF-βs is the type III TGF-β receptor. This proteoglycan is a cell membrane receptor that can act as a reservoir for TGF-β and is also known as betaglycan (or soluble betaglycan if cleaved from the cell membrane and found free in the ECM).

The modulation of the activity of growth factors such as TGF-β is of significant clinical interest. Various parties have investigated the usefulness of proteoglycans as pharmacologically active agents. For instance, the use of such molecules to regulate fibrotic conditions, wound healing and scarring is contemplated in:

(1) WO 93/09800 - relating to the use of decorin and related proteoglycans as agents for preventing or reducing scarring; and

(2) WO 97/05892 - which discloses the use of soluble betaglycan as an anti-scarring agent

The Applicant's co-pending application No. PCT/GB2002/004785 relates to novel modified procollagen molecules wherein at least one N-terminal domain of the molecule contains a polypeptide sequence from at least part of a proteoglycan protein core. The production of collagen gels and matrices from such modified procollagens has been found to assist in wound healing by attracting growth factors to the wound site. Furthermore, the procollagen matrices have been found to have increased resistance to cell shrinkage.

Despite these advances there remains a need to develop further medicaments for assisting in wound healing whilst avoiding or reducing the drawbacks experienced with the prior art applications.

Laminins are a large family of multifunctional glycoproteins which are distributed ubiquitously within basement membranes. The laminins have key roles in development, differentiation and migration due to their ability to interact with cells by means of their high affinity binding sites via cell-surface reactors including integrins and type IV collagen. They are composed of three genetically distinct chains, being $\alpha\beta\gamma$ heterotrimeric proteins that assemble into a cruciform molecule with one long arm and three short arms. There are 18 different laminin isoforms, including Laminin-1, Laminin-2, Laminin-5 and Laminin-10.

The laminins are known to bind keratinocytes and provide survival and differentiation signals to epithelial cells and keratinocytes which are critical cells needed for re-epithelialisation of dermal wounds.

A further molecule that is secreted into the extracellular matrix and is involved in wound healing is secretory leukocyte protease inhibitor (SLPI). This molecule, also known as antileukoprotease, is an 11.7kD cationic inhibitor of neutrophil elastase. In addition to protecting against injury, it has also been shown that it functions as an antimicrobial and anti inflammatory. SLPI is produced naturally by the blood and modifies levels of elastase, a substance which breaks down the skin.

It is an object of the present invention to address problems associated with prior art medicaments and delivery systems. A further object of the present invention is to address problems associated with collagen matrices and gels known in the art.

The present invention is based upon the realisation by the inventors that desirable functional characteristics may be introduced into a composition such as a medicament or collagen matrix by designing modified pro-α chains according to a first aspect of the present invention which may be trimerised to form procollagen derivatives. These in turn may be converted to collagen monomers (with retained propeptides) and subsequently polymerised. This allows the synthesis and assembly of novel collagen polymers having new biological properties.

To this end, a first aspect of the present invention provides a modified pro-α chain comprising a triple helical forming domain linked to at least an N-terminal

domain characterised in that the N-terminal domain contains a polypeptide sequence from at least part of a laminin glycoprotein or at least part of a secretory leukocyte protease inhibitor or functional derivatives thereof.

The inventors have found that they can employ molecular biology techniques to modify the gene encoding pro-α chains such that modified pro-α chains according to the first aspect of the invention may be expressed therefrom. Therefore according to a second aspect of the invention there is provided a DNA molecule encoding modified pro-α chains according to the first aspect of the invention.

The inventors then trimerised modified pro-α chains according to the first aspect of the invention to form a procollagen molecule with a modified N propeptide. The trimer may be a homotrimer of modified pro-α chains or may be a heterotrimer also containing natural pro-α chains. Therefore according to a third aspect of the present invention there is provided a procollagen molecule comprising a trimer of pro-α chains characterised in that at least one of the pro-α chains is a pro-α chain according to the first aspect of the invention.

The inventors then performed further experiments that established that procollagen molecules according to the third aspect of the invention may be polymerised to form a collagen polymer. Furthermore they have established that they can regulate N-propeptide cleavage by modifying the N-terminal domain such that the domain's susceptibility to cleavage is altered such that the collagen polymer retains N-propeptides or derivatives thereof upon its surface. This may be achieved by designing procollagen molecules according to the third aspect of the invention such that they are resistant to procollagen N-proteinases. Alternatively, the molecules may only be partially cleaved or cleaved more slowly. It is preferred that pro-α chains according to the first aspect of the invention are also modified such that they contain an amino acid sequence that confers resistance to procollagen N-proteinases.

Alternatively the inventors have found that they can assemble collagen polymers with retained N-propeptides in an environment in which procollagen N-proteinase is either inhibited or absent.

According to a fourth aspect of the invention there is provided a collagen polymer with at least some of the collagen monomers contained therein having retained N-terminal ends characterised in that at least some of the retained N-terminal ends contain a polypeptide sequence encoding at least part of a laminin glycoprotein, at least part of a secretory leukocyte protease inhibitor or functional derivatives thereof.

Collagen polymers according to the fourth aspect of the invention may form collagen fibrils.

Additionally, the C-terminal domains of the procollagens making up the collagen polymer may be removed, for example using a procollagen C-proteinase, such as bone morphogenetic protein (BMP-1). This has been found to result in the N-terminal propeptides being presented at the fibril surface.

EP-A-0 985 732 contemplates the production of chimeric collagens with biologically active peptides (e.g. a growth factor *per se*) fused to the N-terminal and which can polymerise to form fibrils. However EP-A-0 985 732 does not contemplate or suggest the addition of the polypeptide sequence of at least part of a laminin or secretory leukocyte protease inhibitor (SLPI) to the N terminal domain of a pro-α chain according to the first aspect of the invention.

Modified pro-α chains according to the first aspect of the invention are preferably modified forms of fibrillar forming procollagens (e.g. modified forms of type I, II, III, V or XI pro-α chains). Preferably the molecule is a modified type III pro-α chain. This type is preferred because it can co-assemble with type I collagen and can also form a homotrimer. It is most preferably a modified proα1(III) chain.

It is prefered that only part of a laminin molecule is attached to the pro-α chain. More preferably, the N-terminal ends are derived from the globular domains of an α-chain of a laminin molecule. It is most preferred that the N-terminal end comprises the amino acid sequence for at least the G3 globular domain of the α-chain. Alternatively, the N-terminal may comprise the amino acid sequence for the G1 to G3 domains.

In a preferred embodiment of the invention, the N-terminal sequence of the pro-α chain is replaced with at least part of the amino acid sequence of the α3-chain of Laminin-5 since Laminin-5 has a high affinity for cells of epithelial origin.

In the case of the replacement of the N-terminal end with the polypeptide sequence encoding at least part of a secretory leukocyte protease inhibitor, it is preferred that the entire sequence of the inhibitor is attached to the N-terminal domain.

Preferably, the N-propeptide sequence of the pro-α chain replaces the procollagen N-propeptide sequence prior to N100 to ensure that the collagen retains its signal sequence.

Natural N-terminal propeptide forming domains may be modified such that essentially all of the N-terminal end is replaced by a laminin glycoprotein or SLPI. The extent to which the normal N-terminal propeptide forming domain is replaced is less critical than ensuring that keratinocyte-binding functionality of the laminin molecule or the elastase inhibitory functionality of the SLPI molecule is introduced. Accordingly the N-terminal propeptide forming domain may be totally replaced, partially replaced or even maintained in its entirety (provided it has the required functionality added).

It is desirable to make some modified pro-α chains according to the present invention that trimerise to form procollagens that are resistant to N propeptide cleavage. Therefore some preferred molecules according to the first aspect of the invention have amino acid sequences defining a modified N-proteinase cleavage site

which renders procollagens resistant to such cleavage. People with the Ehlers Danlos syndrome type VII have mutations in a collagen gene which abolishes the N-proteinase cleavage site on the procollagen molecule. Therefore with knowledge of this mutation the region of the domain requiring such modification is easily identified.

The region between the helical forming domain and N-propeptide forming domain of the pro-α chain (the so called hinge domain) is most suitably modified to confer resistance to N-proteinases. For instance, Pro-Gln at the cleavage site may be altered to Leu-Pro.

Modified pro-α chains according to the first aspect of the invention may be formed by direct chemical synthesis or by *in vitro* amino acid polymerization followed by protein folding and, if appropriate, glycosylation of the modified polypeptide sequence. However it is preferred that molecular biology techniques are used to design a DNA molecule according to the second aspect of the invention and express the modified pro-α chain in a cell or expression system containing such a DNA molecule.

The DNA molecule according to the second aspect of the invention may be formed by manipulating the bases encoding the N-terminal propeptide forming domains such that amino acids are added, substituted or deleted. It is preferred that a nucleotide sequence encoding a laminin, SLPI or functional derivatives thereof is inserted into the bases encoding the N propeptide forming domain. It is particularly preferred that a nucleotide sequence encoding at least the G3 domain of the α-chain of a laminin glycoprotein or all of the SLPI molecule is inserted into the bases encoding the N propeptide forming domain.

Preferred modifications include the insertion of a nucleotide sequence encoding the G3 or the G1, G2 and G3 domains of the α-3 chain of Laminin-5.

Alternatively the bases encoding an N propeptide forming domain of a natural pro-α chain may be completely excised and replaced with bases encoding at least one

of the globular domains of an α-chain of laminin or those encoding the SLPI molecule.

According to a preferred embodiment of the invention, the DNA molecule may encode a C-propeptide domain and an α-chain of a pro-α chain and may have the "natural" N-propeptide entirely replaced by a sequence encoding at least one globular domain of an α-chain of a laminin glycoprotein or the SLPI protein.

As previously indicated it is desirable to make some pro-α chains, procollagens or collagen polymers according to the present invention resistant to N propeptide cleavage. Therefore some preferred DNA molecules according to the second aspect of the invention have DNA sequences encoding a modified N-proteinase cleavage site which alters the proteins expressed therefrom resistance to such cleavage. Preferably, the expressed proteins are resistant to cleavage. Alternatively, cleavage in the expressed protein may be partial or slower than in the un-modified protein. It is preferred that the region between the helical forming domain and N-propetide forming domain of the pro-α chain (the so called hinge domain) is mutated to confer resistance to N-proteinases. For instance, nucleotides encoding Pro-Gln at the cleavage site may be altered to nucleotides encoding Leu-Pro.

The DNA molecule may be incorporated within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such vectors will frequently include one or more selectable markers to enable selection of cells transfected with the said vector and, preferably, to enable selection of cells harbouring the recombinant vectors that incorporate the DNA molecule according to the second aspect of the invention.

Standard molecular biology techniques may be used to construct vectors comprising DNA molecules according to the second aspect of the invention. Preferred constructs and expression systems are described in more detail in the Examples.

Vectors may be expression vectors and have regulatory sequences to drive expression of the DNA molecule. Vectors not including such regulatory sequences

may also be used and are useful as cloning vectors for the purposes of replicating the DNA molecule. When such vectors are used the DNA molecule will ultimately be required to be transferred to a suitable expression vector which may be used for production of the procollagen derivative of the invention.

Replication of the DNA molecule in cloning vectors or expression of the protein product from recombinant expression vectors is performed within a suitable host cell. The DNA molecule may be incorporated within a vector within the host cell. Such host cells may be prokaryotic or eukaryotic. Eukaryotic hosts may include yeasts, insect and mammalian cells. Hosts used for expression of the protein encoded by the DNA molecule are ideally stably transformed, although the use of unstably transformed (transient) hosts is not precluded.

A preferred host cell is the HEK293 cell line and derivatives thereof.

The DNA molecule of the invention may also be incorporated in a transgene construct designed for expression in a transgenic plant or, preferably, animal. Transgenic animals which may be suitably formed for expression of such transgene constructs, include birds such as domestic fowl, amphibian species and fish species. The protein may be harvested from body fluids or other body products (such as eggs, where appropriate). Preferred transgenic animals are (non-human) mammals, particularly placental mammals. An expression product of the DNA molecule of the second aspect of the invention may be expressed in the mammary gland of such mammals and the expression product may subsequently be recovered from the milk. Ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs are most suitable placental mammals for use as transgenic animals according to the invention. The generation and usefulness of such mammalian transgenic mammary expression systems is both generally, and in certain instances specifically, disclosed in WO-A-8800239 and WO-9005188.

It is preferred that the host contains suitable intracellular facilities for the assembly of the procollagen derivative of the first aspect of the invention from the protein products of the DNA molecule of the second aspect of the invention. In particular, expression hosts, particularly transgenic animals, may contain other

exogenous DNA the expression of which facilitates the expression, assembly, secretion or other aspects of the biosynthesis of procollagen derivatives of the third aspect of the invention and even collagen polymers according to the fourth aspect of the invention. For example, expression hosts may co-express prolyl 4-hydroxylase, which is a post translation enzyme important in the natural biosynthesis of procollagens, as disclosed in WO-9307889.

DNA, particularly cDNA, encoding natural pro-α chains is known and available in the art. For example, WO-A-9307889, WO-A-9416570 and the references cited in both of them give details. Such DNA may be used as a convenient starting point for making a DNA molecule of the present invention. Recombinant techniques may be used to derive the DNA molecule of the invention from such a starting point.

DNA sequences, cDNAs, full genomic sequences and minigenes (genomic sequences containing some, but not all, of the introns present in the full length gene) may be inserted by recombinant means into a DNA sequence coding for naturally occurring pro-α chains (such as the starting point DNA mentioned above) to form the DNA molecule according to the second aspect of the invention. Because of the large number of introns present in collagen genes in general, experimental practicalities will usually favour the use of cDNAs or, in some circumstances, minigenes. The inserted DNA sequences, cDNAs, full genomic sequences or minigenes code for amino acids which when expressed and assembled into a procollagen according to the third aspect of the invention give rise to a desired modification in the N-terminal domain of such a procollagen derivative.

Any of the DNA material used in these methods (including the DNA sequences, cDNAs, full genomic sequences and minigenes; the DNA molecule according to the second aspect of the invention and vectors) may be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes. However recombinant DNA technology forms the method of choice.

A preferred vector for DNA molecules according to the second aspect of the invention is the episomally replicating plasmid pCep4. This plasmid allows high levels of expression of cloned DNA molecules in cell-lines such as HEK293 transfected with the EBV nuclear antigen.

Collagen polymers in accordance with the fourth aspect of the invention may be of a number of forms. Cylindrical polymers similar to collagen fibrils are generated from mixtures of collagen molecules and collagens derived from procollagens according to the third aspect of the invention when collagen molecules are the major component. Alternatively, sheet-like structures may be formed by using procollagen derivatives according to the third aspect of the invention in the absence of, or substantially in the absence of, normal collagen molecules.

A remarkable feature of collagen polymers according to the fourth aspect of the invention is that the modified N-terminal propeptides are located to the surface of the polymer/fibril so formed, particularly in the case where the C-terminal domain of the procollagen has been removed. The inventors have demonstrated that fibrils formed from mixtures of natural collagens and modified procollagens according to the third aspect of the invention exhibit the modifed N-propeptides at the fibril surface whereas the natural collagens (i.e. those without retained N-propeptides) form the core of the fibril. The arrangement of the molecules in the fibril optimises presentation of the N-propeptides to the interfibrillar space.

Additionally, the inventors were able to form collagen matrices from procollagen molecules according to the third aspect of the invention and/or collagen polymers according to the fourth aspect of the invention. Said collagen matrices form an important fifth aspect of the invention.

Preferably, the matrix is characterised by the fact that at least some of the collagen monomers have a N terminal domain containing at least part of a laminin glycoprotein or at least part of a secretory leukocyte protease inhibitor.

The collagen matrices according to the fifth aspect of the invention have several advantages over known collagen matrices. The incorporation of the globular

domain of a laminin glycoprotein into the collagen matrix promotes keratinocyte crawling due to their keratinocyte binding properties and thereby accelerate re-epithelialisation. Thus, the matrices may be used to recruit viable cells from wound margins.

Furthermore, the incorporation of SLPI domain into the collagen matrices also aids wound healing, provides anti-microbial and antiflammatory properties and reduces breakdown of the skin.

Collagen matrices according to the fifth aspect of the invention are preferably made from human recombinant DNA molecules according to the second aspect of the invention. When this is the case, a third advantage is that the matrices are less likely to cause allergic and inflammatory responses when administered to humans.

A collagen matrix may be formed by neutralising and warming acidic solutions of collagen monomers or procollagens (in the presence of suitable proteinases). Under such conditions the collagen monomers spontaneously self-assemble into polymeric fibrils that then become entangled to form a hydrated and porous gel. The rigidity of such a gel is, at least in part, dependent on the concentration of the collagen used to form the gel and on the diameter of the collagen fibrils formed. The collagen matrix or gel assumes the shape of the container in which it is formed. Therefore, gels can be made that are thin (millimetres) in one dimension and extensive (centimetres or larger) in other dimensions. Such matrices can be suitably shaped to form the basis of replacement skin or cornea. Alternatively, collagen gels can be cast in moulds that have the shape of long bones (cylindrical and long), jaw bones (sickle shaped or curved), articular cartilage (disc shaped), tendon (rope shaped) or ligament (shaped like a strap).

Collagen polymers and matrices according to the fourth and fifth aspects of the invention may comprise exclusively recombinant collagen derived from modified procollagen molecules according to the invention. Alternatively such collagen polymers or matrices may be mixtures of modified collagens or modified procollagens according to the invention and collagen extracted from tissue or cell cultures, such as is available from commercial sources. For example, collagen

polymers according to the fourth aspect of the invention may be combined with bovine type I collagen to form a matrix according to a fifth aspect of the invention.

Procollagens or collagens according to the present invention may be used to coat the surfaces of collagen fibrils in a gel or matrix formed from natural collagens (e.g bovine collagens) or they may be incorporated into the fibrils during gel formation. The new functional moieties introduced into the procollagens or collagens are thereby presented to the surface of the collagen fibrils where they can interact with cells or influence cellular function. The procollagens may be applied as a soluble precursor with a procollagen C-proteinase such as BMP-1 which converts the soluble procollagen to fibril-forming collagen having its N-terminal domain retained to allow gel formation *in situ*. This enables the modified collagen to integrate and mesh with collagen fibrils at the point of application.

Molecules according to the first – fifth aspects of the invention may be employed in a research setting for exploring a wide range of biological phenomenon from cell adhesion to wound healing and from cell differentiation and apoptosis to the manufacture of wound dressings with improved molecule and cell binding properties. However, a preferred use of the molecules is in the formation of collagen matrices which may be used for medical or cosmetic purposes.

According to a sixth aspect of the present invention there is provided the use of a molecule or matrix according to any one of the first - fifth aspects of the invention for the treatment of medical conditions.

According to a seventh aspect of the present invention there is provided the use of a molecule or matrix according to any one of the first - fifth aspects of the invention for the manufacture of a medicament for use in the treatment of wounds or fibrotic disorders.

According to a eighth aspect of the present invention there is provided a method of treating wounds comprising administering to a subject in need of treatment a therapeutically effective amount of a molecule or matrix according to any one of the first – fifth aspects of the invention.

It is preferred that the medical conditions treated are conditions that are at least partially characterised by remodelling of the ECM.

Whilst the above considerations mainly apply to conditions, disorders or diseases of man it will be appreciated that wound healing can also be problematic in other animals, particularly veterinary or domestic animals (e.g. horses, cattle, dogs, cats etc). For instance abdominal wounds or adhesions are a major reason for having to put down horses (particularly race horses), as are tendon and ligament damage leading to scarring or fibrosis.

Molecules according to the third and fourth aspects of the invention and a matrix according to the fifth aspect of the invention may be formulated into a various types of medicament. The medicament of the invention may take a number of different forms depending, in particular on the manner in which the medicament is to be used. Thus, for example, the medicament may be in the form of a liquid, ointment, cream, gel, hydrogel, powder, aerosol or an implantable device (e.g. by conjugation to a biopolymer sponge).

Molecules according to the third and fourth aspects of the invention may be administered directly (e.g. in liquid form). However, it is preferred that the molecules are incorporated into a wound dressing, an implantable device, artificial skin or tissue etc.

It is preferred that the medicaments are for topical application. The medicament may be most suitably used for topical application to the skin or wound area.

Medicaments comprising modified procollagens, collagens or collagen fibrils may be delivered by means of an aerosol (e.g. for delivery to fibrotic conditions of the lung).

It will be appreciated that the vehicle of the medicament should be one which is well tolerated by the patient and allows release of the collagen polymer to the wound or site of fibrosis. The vehicle will ideally be sterile and may be combined with

excipients and / or stabilizers as well as the molecule to form the medicament. Such a vehicle is preferably biodegradeable, bioresolvable, bioresorbable and/or non-inflammatory.

The medicament may be used in a number of ways. Thus, for example, it may be applied in, and/or around a wound of a patient to provide the desired promotion of wound healing. If the composition is to be applied to an "existing" wound, then the pharmaceutically acceptable vehicle will be "mild" enough such that it does not cause an inflammatory response or is toxic to the tissue. Clearly, the inclusion of modified collagen containing the SLPI molecule will assist in reducing any inflammatory response.

Molecules according to the third or fourth aspects of the invention may be provided on a sterile dressing or patch which may be used to cover or even pack a wound or fibrotic site.

The medicament may be provided as an implantable device from which it may be released better. For instance, it may be released by biological dissolution or degradation of the device. Alternatively an external stimulus, such as ultrasound, may cause release of the procollagen, collagen monomer or collagen polymer.

It is also possible to use medicaments in accordance with the invention in a prophylactic manner. For instance, the medicament may be applied prior to surgery so as to provide for regulation of healing of the subsequently formed surgical wound.

A collagen matrix may then be administered to a subject (e.g. to the skin, cartilage, muscle or neural tissues) in the form of a semi-solid gel. Alternatively a more solid matrix may be formed which may be used in the formation of a wound dressing, an implantable device, artificial skin or tissue etc.

Artificial skins comprising matrices according to the fifth aspect of the invention may comprise ECM components alone or may further comprise cultured cells such as fibroblasts and/or endothelial cells. Artificial skins containing such cells are known as "living" replacement skin products.

It is preferred that the collagen matrices are formed into artificial skin for topical application to dermal wounds or burns. The artificial skins comprising matrices according to the fifth aspect of the invention are particularly useful for treating severe wounds, extensive wounds, chronic wounds (e.g. dermal ulcers) and burns.

It will be appreciated that the matrix should be hydrated in a pharmaceutically acceptable vehicle. The vehicle should be sterile and "mild" enough such that it does not cause an inflammatory response or is toxic to the tissue being treated.

The matrix may be incorporated into a sterile dressing or patch which may be used to cover or even pack a wound or fibrotic site.

In a preferred embodiment, the matrix is applied to a dressing, such as a Combiderm N dressing and then dehydrated. The dehydrated gel carried on the dressing is then applied to a wound.

The matrix may be provided as an implantable device from which the matrix *per se* may be released into the wound site. Release may be caused by biological dissolution or degradation of the device. Alternatively an external stimulus, such as ultrasound, may cause release of the collagen polymer.

A collagen matrix according to the fifth aspect of the invention may be cast into a sheet. Preferred sheets may be 1- several millimetres thick by several centimetres square. Such sheets can be acellular or populated with mesenchymal and/or fibroblastic cells to generate an artificial skin, cartilage, bone or cornea, or endothelial cells to produce cardiovascular patches. The cells may be obtained from a patient or a tissue-matched donor, stem cells from a patient or a donor, or cells that have been amplified in culture. Such matrices may be coated with molecules according to the third and fourth aspects of the invention to confer keratinocyte binding functionality or elastase inhibition to the matrix. The collagen matrix or collagen-cell construct can be stored under aseptic conditions and at physiological temperatures or under cryogenic storage conditions until needed.

It will be appreciated that the amount of molecule required to modulate healing and fibrosis depends on a number of factors such as its biological activity and bioavailability, which in turn depends on the mode of administration and the physicochemical properties of the particular molecule used. For example, the amount of collagen matrix required will depend upon factors such as the concentration of the gel (this may be required to be aqueous, viscous or relatively solid – depending upon the clinical need) and the proportion of collagens with the new functional moieties contained therein. Other factors include:

A) The specific condition to be treated.

B) The severity of the condition.

C) The age of the subject.

D) The site of delivery.

E) The half-life of the molecule in the subject being treated.

The frequency of administration will also be influenced by the above mentioned factors and particularly the half-life of the compound or matrix within the subject being treated.

Generally, a subject being treated will derive benefit from the application of the modified procollagen, collagen monomer or collagen polymer, if it as administered to a wound within 7 days of wounding, preferably within 48 hours of wounding, more preferably within 24 hours of wounding and even more preferably within 12 hours of wounding. The medicament should be administered to a subject suffering from a fibrotic condition according to a clinicians directions. This may be as soon as diagnosis has occurred. Therapy should continue until the wound has healed or fibrotic disorder cleared to a clinicians satisfaction.

When used as a prophylactic (e.g. before surgery) the medicament should be administered as soon as it is recognised that a wound may occur or fibrotic disorder may develop. For instance, a cream or ointment containing collagen polymer according to a fourth aspect of the invention may be applied to a site on the skin of a subject where elective surgery is to be performed and an increased rate of wound healing is subsequently desired. In this case, the medicament may be applied during

the preoperative preparation of the subject or it may even be desirable to apply it in the hours or days preceding the surgery (depending upon the health status and age of subject as well as the size of the wound to be formed).

Frequency of administration will depend upon the biological half-life of the molecule used. Typically a cream or ointment should be administered to a target tissue such that the concentration of the molecule at the wound site is maintained at a level suitable for having a therapeutic effect. This may require administration daily or even several times daily.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of compositions and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration).

Generally, for use in accordance with the invention a medicament containing an amount of 1ng to 10mg of collagen polymer, more preferably 1μg to 1mg of collagen polymer, may be applied per centimetre of linear wound. Purely by way of example, a medicament containing about 10μg collagen polymer is suitable for application to a 1 cm linear incisonal wound. Higher doses are required to stimulate the healing of chronic wounds compared to acute wounds.

Efficacy of medicaments, and particularly those formulated for application to chronic wounds, have enhanced efficacy when combined with a protease inhibitor (e.g. galadrin) Protease inhibitors prevent or retard the degradation of the collagen by proteases which may be found in high levels in wounds, particularly chronic wounds. The protease inhibitor is preferably a broad spectrum protease inhibitor.

It will be appreciated that the molecules and matrices according to the third, fourth and fifth aspects of the invention may be used in combination with other wound healing or anti-fibrotic agents or followed by another agent (e.g. for prevention of scarring).

It will be appreciated that matrices according to the fifth aspect of the invention (used to treat medical conditions, cosmetically or otherwise) may be formed *in situ* (i.e. at the tissue/site where the matrix is required). For instance, a solution or slurry of collagen polymers according to the fourth aspect of the invention may be used to soak a wound dressing. Gel formation may be induced when the dressing is used (e.g. a reaction may initiated when the dressing is removed from its package or contacts a wound site). Alternatively a solution of collagen polymers according to the fourth aspect of the invention, or even procollagens according to the third aspect of the invention may be injected into a target body tissue and matrix formation allowed to proceed with native collagens.

DNA molecules according to the second aspect of the invention may be used in gene therapy techniques. Therefore according to a ninth aspect of the present invention there is provided a delivery system for use in a gene therapy technique, said delivery system comprising a DNA molecule according to the second aspect of the invention which is capable of being transcribed to lead to the expression of a modified pro-α chain according to the first aspect of the invention at a wound site or site of fibrosis.

According to a tenth aspect of the present invention there is provided the use of a delivery system as defined in the preceding paragraph for use in the manufacture of a medicament for treating wounds or fibrotic disorders.

According to an eleventh aspect of the present invention there is provided a method of treating a wound or fibrotic condition which consists of administering to a patient in need of treatment a therapeutic dose of a delivery system as defined above.

The delivery systems are highly suitable for achieving sustained levels of a procollagen molecule according to the third aspect of the invention or a collagen polymer according to the fourth aspect of the invention at a wound site or site of fibrosis over a longer period of time than is possible for most conventional delivery systems. Modified pro-α chains may be continuously expressed from cells at the site that have been transformed with the DNA molecule of the second aspect of the

invention. Therefore, even if the modified procollagen or collagen polymer has a very short half-life as an agent *in vivo*, therapeutic doses may be continuously expressed from the treated tissue.

Furthermore, the delivery system of the invention may be used to provide the DNA molecule without the need to use conventional pharmaceutical vehicles such as those required in ointments or creams that are contacted with the wound or site of fibrosis. This is particularly beneficial as it can often be difficult to provide a satisfactory vehicle for a compound for use in wound healing (which are required to be non-inflammatory, biocompatible, bioresorbable and must not degrade or inactivate the active agent (in storage or in use)).

The delivery system is such that the DNA molecule is capable of being expressed (when the delivery system is administered to a patient) to produce modified pro-α chains which form procollagens and then collagen polymers with the modified N terminals. These modified N terminals then interact with cells or biologically active agents at the site of the wound or fibrosis and thereby treat the condition.

The DNA molecule may be contained within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such recombinant vectors are highly useful in the delivery systems of the invention for transforming cells with the DNA molecule. The vector may be pCEP4 or a similar vector.

Recombinant vectors may also include other functional elements. For instance, recombinant vectors can be designed such that the vector will autonomously replicate in the nucleus of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. Alternatively the recombinant vector may be designed such that the vector and recombinant DNA molecule integrates into the genome of a cell. In this case DNA sequences which favour targeted integration (e.g. by homologous recombination) are desirable. Recombinant vectors may also have DNA coding for genes that may be used as selectable markers in the cloning process.

The recombinant vector may also further comprise a promoter or regulator to control expression of the gene as required.

The DNA molecule may (but not necessarily) be one which becomes incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed leading to the production of genetically modified daughter cells (in which case regulation of expression in the subject may be required e.g. with specific transcription factors or gene activators). Alternatively, the delivery system may be designed to favour unstable or transient transformation of differentiated cells in the subject being treated. When this is the case, regulation of expression may be less important because expression of the DNA molecule will stop when the transformed cells die or stop expressing the protein (ideally when the wound, fibrosis or scarring has been treated or prevented).

The delivery system may provide the DNA molecule to the subject without it being incorporated in a vector. For instance, the DNA molecule may be incorporated within a liposome or virus particle. Alternatively the "naked" DNA molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The DNA molecule may be transferred to the cells of a subject to be treated by transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the DNA molecule, viral vectors (e.g. adenovirus) and means of providing direct DNA uptake (e.g. endocytosis) by application of plasmid DNA directly to the wounded area topically or by injection.

Whilst the above considerations mainly apply to wounds of man it will be appreciated that wound healing, can also be problematic in other animals (especially veterinary and domestic animals such as cattle, horses, dogs, cats etc). For instance, abdominal wounds or adhesions are a major reason for having to put down horses. The medicaments and delivery systems discussed above are also suitable for use in the healing of such animals.

The present invention will now be further described with reference to the following non-limiting examples and figures in which:

Figure 1 schematically illustrates a natural procollagen molecule;

Figure 2 schematically illustrates lam-procollagen, a procollagen molecule according to the third aspect of the invention;

Figure 3 illustrates the nucleotide sequence of a DNA molecule according to the second aspect of the invention from Example 1;

Figure 4 illustrates the amino acid sequence of a modified pro-α chain according to the first aspect of the invention from Example 1;

Figure 5 is a photograph of a Western blot referred to in Examples 1 and 2;

Figure 6 illustrates the nucleotide sequence of a DNA molecule according to the second aspect of the present invention from Example 2;

Figure 7 illustrates the amino acid sequence of a modified pro-α chain according to a first aspect of the invention from Example 2;

Figure 8 illustrates the nucleotide sequence of a DNA molecule according to the second aspect of the invention from Example 3;

Figure 9 illustrates the amino acid sequence of a modified pro-α chain according to the first aspect of the invention from Example 3; and

Figure 10 is a photograph of a Western Blot referred to in Example 3.

Figure 1 illustrates a natural procollagen with an N-terminal propeptide 1, alpha helical domain 2 and a C-terminal propeptide 3. A procollagen N-Proteinase cleavage site 4 in the hinge region of the molecule (between 1 and 2) is also illustrated. Figure 2 illustrates lam-proα1(III) or Lam-Coll™ a procollagen molecule according to the third aspect of the invention in which the N propeptide 1 has been replaced by at least one globular binding domain of laminin 5.

EXAMPLE 1: Design and Construction of a DNA molecule according to the second aspect of the invention, the Amino Acid sequence of the modified pro-α chain expressed therefrom according to a first aspect of the invention and the expression and characterisation of modified procollagens prepared therefrom according to a third aspect of the invention.

A DNA molecule according to the second aspect of the invention was constructed comprising the entire coding region for the G1, G2 and G3 domains of the α-3 chain of Laminin 5 in place of the globular domain of the N-propeptide of the proα1(III) chain.

The cloning strategy for production of the DNA molecule involved the following primary PCR reactions.

1. Substrate : pRMI containing the complete cDNA for pro-α1 (III) chain of collagen (publicly available X 14420).

Oligonucleotides:

T3 (5' end) 5' AATTAACCCTCACTAAAGGG 3'

(Seq ID.No.1)

SSG1-20R

(3' end) 5' ACAGAGATGTTGCCAAAATAATAGTGGGATG 3'

(Seq ID No. 2)

Product A: 300 bp.

2. Substrate: Lam5α3-pSECTAG2C containing gene for α3 chain cloned in on Asp718I site

Oligonucleotides:

SSG1-20F(5'end)

5' TATTTTGGCAACATCTCTGTCCTTGTTTCTC 3'

(Seq. ID No. 3)

LG3-20R (3' end)

5' CTTGACCATTAGCATCTTGCCACACCTTCAC 3'

(Seq. ID No. 4)

Product B: 1800 bp

3. Substate: pRM1

Oligonucleotides:

LG3-20F (5' end)

5' GCAAGATGCTAATGGTCAAGGACCTCAAGGC 3'

(Seq ID No. 5)

III-JL11 (3' end)

5' AGACCCTGCAGGTCCAACTT 3' (Seq ID No. 6)

Product C: 700 bp.

The following secondary PCR Reactions were then carried out.

1) Substrate: Mixture of A (300bp) and (B (1.8kb) products

Oligonucleotides: T3 (5'end)

LG3-20R (3'end)

Product AB: 2.1kb

2) Substrate: Mixture of B (1.8kb) and C(700bp)

Oligonucleotides: SSG1-20F (5'end)

III-JL11 (3'end)

Product BC: 2.5kb

Cloning of AB and BC products into pBluescript

Product AB was digested with HindIII and Not1 and then ligated into pBS also digested with HindIII and Not1 to generate G123AB-pBS plasmid. Product BC was digested with HindIII and BAMH1 and then ligated into pBS also digested with HindIII and BAMH1 to generate G123BC-pBS plasmid.

Generation of chimeric LamG123-collagen gene

The G123AB-pBS plasmid was digested with Not1 and HindIII and the 1.27kb fragment was gel purified. The G123BC-pBS plasmid was digested with BamH1 and HindIII and the 1.36kb fragment was gel purified. The pRMI plasmid was digested with Not1 and BamH1 and the 6.8kb fragment was gel purified.

The three fragments were ligated together to generate the gene encoding the LamG123-collagen fusion protein. Correct assembly of the lamG123-collagen gene was determined by DNA sequencing.

Modification of the LamG123-collagen/pBluescript plasmid

A NotI site was introduced 3' to the collagen sequence by standard PCR mediated site-directed mutagenesis using the oligonucleotides TAS14NotA and Oligo32merTAS12NotS, details of which are as follows:-

TAS14NotA (antisense)

5' GTTGTAAAACGGCGGCCGCTGAATTGTAATAC 3'

(Seq ID No. 7)

Oligo32merTAS12NotS (sense)

5' GTATTACAATTCAGCGGCCGCCGTTTTACAAC 3'

(Seq ID No. 8)

The oligonucleotides introduce a NotI site within the pBluescript sequence about 50bp downstream of the KpnI site.

Subcloning into pCEP4

The LamG123-collagen/pBluescript plasmid was digested with NotI to give a 6kb fragment, which was ligated into NotI digested & phosphatased pCEP4 (10.4kb). pCep4 vector (Invitrogen Life Technologies) is commercially available and the sequence may be found at http://www.invitrogen.com. Correct orientation of the 6kb NotI fragment into pCep4 was determined by DNA sequencing .

Using the cloning strategy outlined above the procollagen type III N-propeptide Sequence prior to N100 was replaced with the sequence for the G123 domains of the α3 chain of Laminin-5, whilst retaining the collagen III signal sequence. The entire nucleotide sequence of the DNA molecule is presented in Fig 3 (and SEQ ID No. 9). Fig.4 (and SEQ ID No. 10) represents the amino acid sequence of the modified pro-α chain (a molecule according to the first aspect of the invention) which may be expressed from the DNA molecule. The junction between the G123 of laminin and procollagen sequences is shown as underlined in Figs 3 and 4.

The DNA molecule sub-cloned into the expression vector PCEP4 was expressed in HEK293-EBNA cells (Invitrogen Life Technologies).

HEK293-EBNA cells are known to those skilled in the art and details are available from http://www.invitrogen.com/Content/Tech-Online/molecular_biology/manuals_pps/293ebna_man.pdf

HEK293-EBNA cells do not secrete procollagens and so are ideal for a negative background to express collagens in. Importantly, these cells do contain prolyl 4-hydroxylase which is vital for the hydroxylation of proline residues in the procollagen sequence and hence for the stability of the triple helix. The HEK293-EBNA line also expresses the EBNA-1 antigen that ensures that any plasmid DNA transfected into the cell is maintained episomally when the presence of that plasmid is selected for by the appropriate antibiotic (generally hygromycin).

Modified pro-α chains according to the first aspect of the invention are generated in the endoplasmic reticulum of the HEK293-EBNA cells. These molecules then automatically form a homotrimer (modified procollagen molecules according to the third aspect of the invention). The modified procollagen molecule produced from said cells is hereinafter referred to LamG123-coll.

A Integra CL 350 flask was seeded with HEK293-EPNA cells transformed with the DNA molecule and left for 7 days. The enriched medium was then harvested three times weekly (days 7, 9, 12, 14 and 16 after seeding).

LamG123-coll was characterised by Western blotting using an anti-collagen antibody. The results are presented in Figure 5 of the accompanying drawings wherein Lane 1 is type III procollagen control, Lane 2 has medium from untransfected 293 cells, Lane 3 has medium from 293 EBNA cells transfected with LG123-coll and Lane 4 has medium from 293 EBNA cells transfected with LamG3-coll (see Example 2 below).

EXAMPLE 2: Design and Construction of a DNA molecule according to the second aspect of the invention, the Amino Acid sequence of the modified pro-α chain expressed therefrom according to a first aspect of the invention and the expression and characterisation of modified procollagens prepared therefrom according to a third aspect of the invention.

A DNA molecule according to the second aspect of the invention was constructed comprising the coding region for the G3 domain of the α-3 chain of Laminin 5 in place of the globular domain of the N-propeptide of the proα1(III) chain.

The cloning strategy for production of the DNA molecule involved the following primary PCR reactions.

1. Substrate : pRMI containing the complete cDNA for pro-α1 (III) chain of collagen (publicly available X 14420).

Oligonucleotides:

T3 (5' end) 5' AATTAACCCTCACTAAAGGG 3'

(Seq ID.No.1)

SSLAMG3-2

(3' end)

5' GCTTCCAGTCTTCCGAGCATGCCAAAATAATAGTGGG 3'

(Seq ID No. 11)

Product A: 300 bp.

2. Substrate: Lam5α3-pSECTAG2C containing gene for α3 chain cloned in on Asp718I site

Oligonucleotides:

SLAMG3-1(5'end)

5' CCCACTATTATTTTGGCATGCTCGGAAGACTGGAAGC 3'

(Seq. ID No. 12)

LG3-20R (3' end)

5' CTTGACCATTAGCATCTTGCCACACCTTCAC 3'

(Seq. ID No. 4)

Product B: 700 bp

The following secondary PCR Reaction was then carried out.

1) Substrate: Mixture of A (300bp) and B (700 bp) products

Oligonucleotides: T3 (5'end)

LG3-20R (3'end)

Product AB: 1.0kb

Cloning of AB product into pBluescript

Product AB was digested with HindIII and Not1 and then ligated into pBS also digested with HindIII and Not1 to generate G3AB-pBS plasmid.

Generation of chimeric LamG-collagen gene

The G3AB-pBS plasmid was digested with Not1 and HindIII and the 200bp fragment was gel purified. The G3AB-pBS plasmid was digested with BamH1 and HindIII and the 1.36kb fragment was gel purified. The pRMI plasmid was digested with Not1 and BamH1 and the 6.8kb fragment was gel purified.

The three fragments were ligated together to generate the gene encoding the LamG3-collagen fusion protein. Correct assembly of the lamG3-collagen gene was determined by DNA sequencing.

Modification of the LamG3-collagen/pBluescript plasmid

A NotI site was introduced 3' to the collagen sequence by standard PCR mediated site-directed mutagenesis using the oligonucleotides TAS14NotA and Oligo32merTAS12NotS, (see Example 1 above)

The oligonucleotides introduce a NotI site within the pBluescript sequence about 50bp downstream of the KpnI site.

Subcloning into pCEP4

The LamG3-collagen/pBluescript plasmid was digested with NotI to give a 5kb fragment, which was ligated into NotI digested & phosphatased pCEP4 (10.4kb). Correct orientation of the 5kb NotI fragment into pCep4 was determined by DNA sequencing .

Using the cloning strategy outlined above the procollagen type III N-propeptide Sequence prior to N100 was replaced with the sequence for the G3 domain of the $\alpha 3$ chain of Laminin-5, whilst retaining the collagen III signal sequence. The entire nucleotide sequence of the DNA molecule is presented in Fig 6 (and SEQ ID No. 13). Fig.7 (and SEQ ID No. 14) represents the amino acid sequence of the modified pro-α chain (a molecule according to the first aspect of the invention) which may be expressed from the DNA molecule. The junction between the G3 of laminin and procollagen sequences is shown as underlined in Figs 6 and 7.

The DNA molecule sub-cloned into the expression vector PCEP4 was expressed in HEK293-EBNA cells (Invitrogen Life Technologies).

Modified pro-α chains according to the first aspect of the invention are generated in the endoplasmic reticulum of the HEK293-EBNA cells. These molecules then automatically form a homotrimer (modified procollagen molecules according to the third aspect of the invention). The modified procollagen molecule produced from said cells is hereinafter referred to LamG3-coll.

A Integra CL 350 flask was seeded with HEK293-EPNA cells transformed with the DNA molecule from this Example and left for 7 days. The enriched medium was then harvested three times weekly (days 7, 9, 12, 14 and 16 after seeding).

LamG3-coll was characterised by Western blotting using an anti-collagen antibody. The results are presented in Figure 5 wherein Lane 4 has medium from 293 EBNA cells transfected with LamG3-coll.

EXAMPLE 3: Design and Construction of a DNA molecule according to the second aspect of the invention, the Amino Acid sequence of the modified pro-α chain expressed therefrom according to a first aspect of the invention and the expression and characterisation of modified procollagens prepared therefrom according to a third aspect of the invention.

A DNA molecule according to the second aspect of the invention was constructed comprising the entire coding region for secretory leukocyte protease inhibitor precursor ("SLPI") in place of the globular domain of the N-propeptide of the proα1(III) chain. "SLPI-Collagen" (or slpi-coll) was produced by constructing the SLPICollagenIII/pCEP4 construct, involving polymerase chain reactions, restriction digestion and ligation.

Polymerase Chain Reactions

The Platinum® Pfx DNA polymerase (Invitrogen, U.K.), the corresponding recipe and cycling programme as recommended by the manufacturer were used for all the PCRs carried out in cloning SLPI-Collagen. Three rounds of PCR were required for the assembly of SLPI-CollagenIII/pCEP4 construct.

In the first round, the sequence encoding human SLPI was amplified from the image clone 4733996 (UK Human Genome Mapping Project Resource Centre, U.K.). The following primers were employed in the PCR:

5' primer 5' - CTTGTAGAT**GCGGCCGC**atgaagtccagcggcctctt-3' (Seq ID No. 15)

3' primer 5'-*cttcaacagc*agctttcacaggggaaacgc-3' (Seq ID No. 16)

The primers resulted in the SLPI PCR products containing a Not I restriction site (GCGGCCGC) at the 5' end, indicated by bold capital letters in the sequence above, and at its 3' end, there were 10 base pairs encoding the 5' end of human type III collagen, indicated by italic small letter in the sequence above. The annealing temperature was 48°C. The PCR product was expected to have a size of 0.42 kilo basepairs (kbp). It was then gel purified using Qiagen Gel Extraction Kit (Qiagen, U.K.).

In the second round of PCR, part of the sequences encoding human type III collagen was amplified from the construct pRMI using the following primers:

5' primer 5'-*tgtgaaagct*gctgttgaaggaggatgttc-3' (Seq ID No. 17)

3' primer 5'-ggacctggtcgaccactttc-3' (Seq ID No. 18)

The italic small letters indicate nucleotides encoding SLPI. The annealing temperature was 50°C. pRMI is a pBluescript SK (-) vector carrying a human type III collagen insert. As a result, the 5' end of the PCR product had 10 base pairs encoding the 3' end of SLPI. The expected size of the Collagen III PCR product was 1.603 kbp. It was then gel purified using Qiagen Gel Extraction Kit (Qiagen).

In the third round of PCR, the sequences encoding SLPI-Collagen III fragment were amplified from the purified SLPI and Collagen III PCR products. The following primers were used:

5' primer	5'-*tgtgaaagct*gctgttgaaggaggatgttc-3'	(Seq ID No. 17)
3' primer	5'-ggacctggtcgaccactttc-3'	(Seq ID No. 18)

The resulting PCR product was expected to have a size of 2.023 kbp. It also contained a Not I and a Xma I restriction sites. It was then gel purified by the Qiagen gel extraction kit.

Restriction, digestion and ligation.

The purified SLPI-Collagen III PCR product was digested with restriction enzymes (Roche, U.K.) Not I and Xma I while the vector pRMI was digested with Not I and EcoR V followed by Xma I. The digests were then gel purified by the Qiagen gel extraction kit and this was followed by the dephosphorylation of the vector digest with alkaline phosphatase. Upon assessing the yield of the inserts and the dephosphorylated vector, a ligation reaction was set up using high concentration T4 DNA ligase (New Englands Biolabs, U.K.), according to manufacturer's instruction.

Transformation and colony screening.

5μl of the ligation reaction was transformed into the chemically competent DH5α cells. The DNA from each colony was extracted by Qiagen miniprep kit (Qiagen). A positive clone was distinguished by restriction digestion with Xho I, yielding fragments of the right sizes on the agarose gel (1.936, 2.520 and 4.680 kbp).

Sequencing of the PCR product.

Once a positive clone was identified, sequencing reactions were carried out to ensure that no error was introduced into the PCR product by the polymerase. The primers used in the sequencing reaction are shown below:

SK-T7	5'-gta ata cga ctc act ata ggg c-3'	(Seq ID No.19)
C3For1	5'-gct gtt gaa gga gga tgt-3'	(Seq ID No.20)

C3For2	5'-aga ggc ttc gat gga cga-3'	(Seq ID No.21)
C3For3	5'gga ctg cga ggt ggt gca-3'	(Seq ID No. 22)
C3Rev1	5'-ttc tcc cag gaa tac cag-3'	(Seq ID No.23)
C3Rev2	5'-agg gaa tcc ggc agt tcc-3'	(Seq ID No. 24)
C3Rev3	5'-ctc ggg gac cag atg gcc-3'	(Seq ID No.25)

Subcloning of SLPI-Collagen III PCR Product into pCEP4.

In order to subclone SLPI-Collagen III into pCEP4, SLPI-Collagen III/SK (+) was digested with Not I. This was followed by filling ends with Klenow (Roche) and restriction digestion with Hind III. The same procedures were performed on the vector pCEP4 except Not I was substituted by Kpn I. The insert and vector were then gel purified using the Qiagen gel extraction kit (Qiagen). In order to prevent self-ligation, the vector was also dephosphorylated with alkaline phosphatase (Roche).

A ligation react[1]ion using high concentration T4 DNA ligase (NEB) was set up after the yields of the insert and vector were assessed. Chemically competent DH5α cells were then transformed with the construct. The DNA from each colony was extracted with Qiagen miniprep kit (Qiagen). Upon digestion with Xho I, a positive clone was revealed by the sizes of the DNA fragments obtained (1.924, 2.520 and 11.480 kbp).

Using the cloning strategy outlined above the procollagen type III N-propeptide sequence was replaced with the sequence for SLPI whilst retaining the collagen III signal sequence. The entire nucleotide sequence of the DNA molecule is presented in Figure 8 (and SEQ ID No. 26). Figure 9 (and SEQ ID No. 27) represents the amino

acid sequence of the modified pro-α chain (a molecule according to the first aspect of the invention) which may be expressed from the DNA molecule. The underlined sections in Figures 8 and 9 relate to the DNA and amino acid sequence of SLPI respectively, whilst the non-underlined sections refer to DNA and amino acid sequences for human procollagen III starting from the von Willebrand Factor.

The DNA molecule cloned into the pCEP4 vector was expressed in HEK 293 Ebna cells, see Figure 10. The band for slpi-col is the single band in the western blotted into anti-slpi antibody.

The above Examples illustrate that modified collagens may be produced that contain part or all of a laminin or SLPI molecule. These modified domains are able to impart specific desirable functional characteristics to the collagen to enhance the wound healing properties of the molecule.

FIG. 1

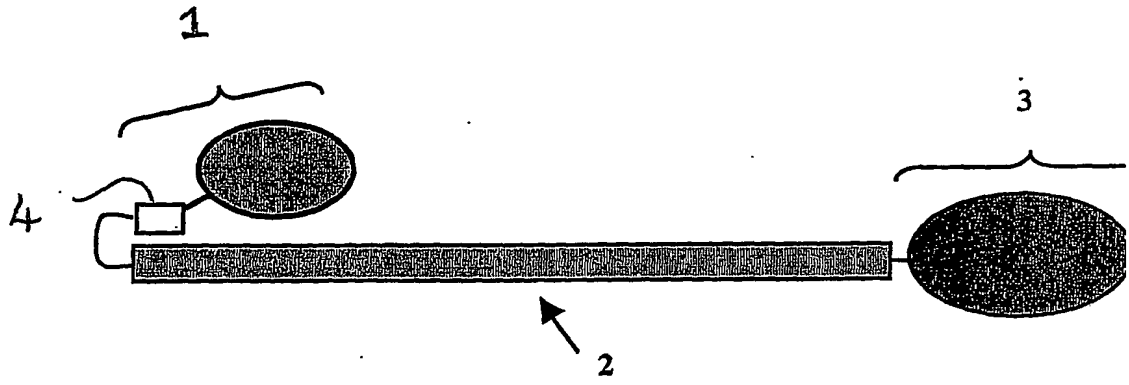

FIG. 2

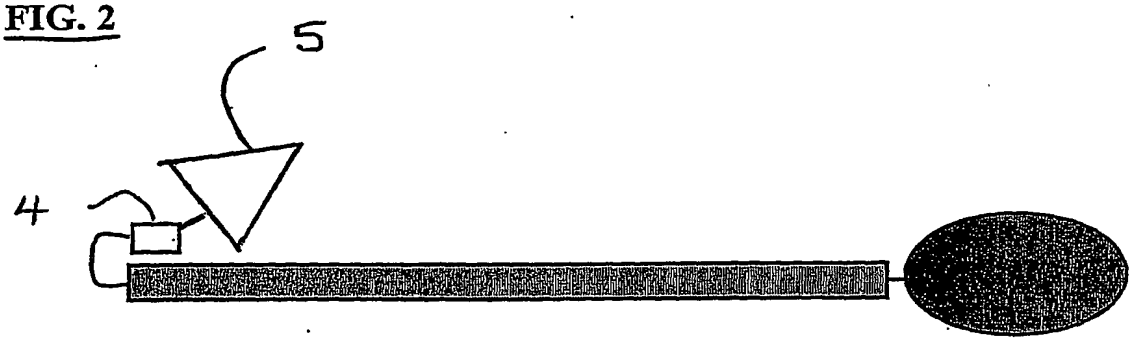

Fig 3.

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Fig. 3 cont;

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Fig. 4

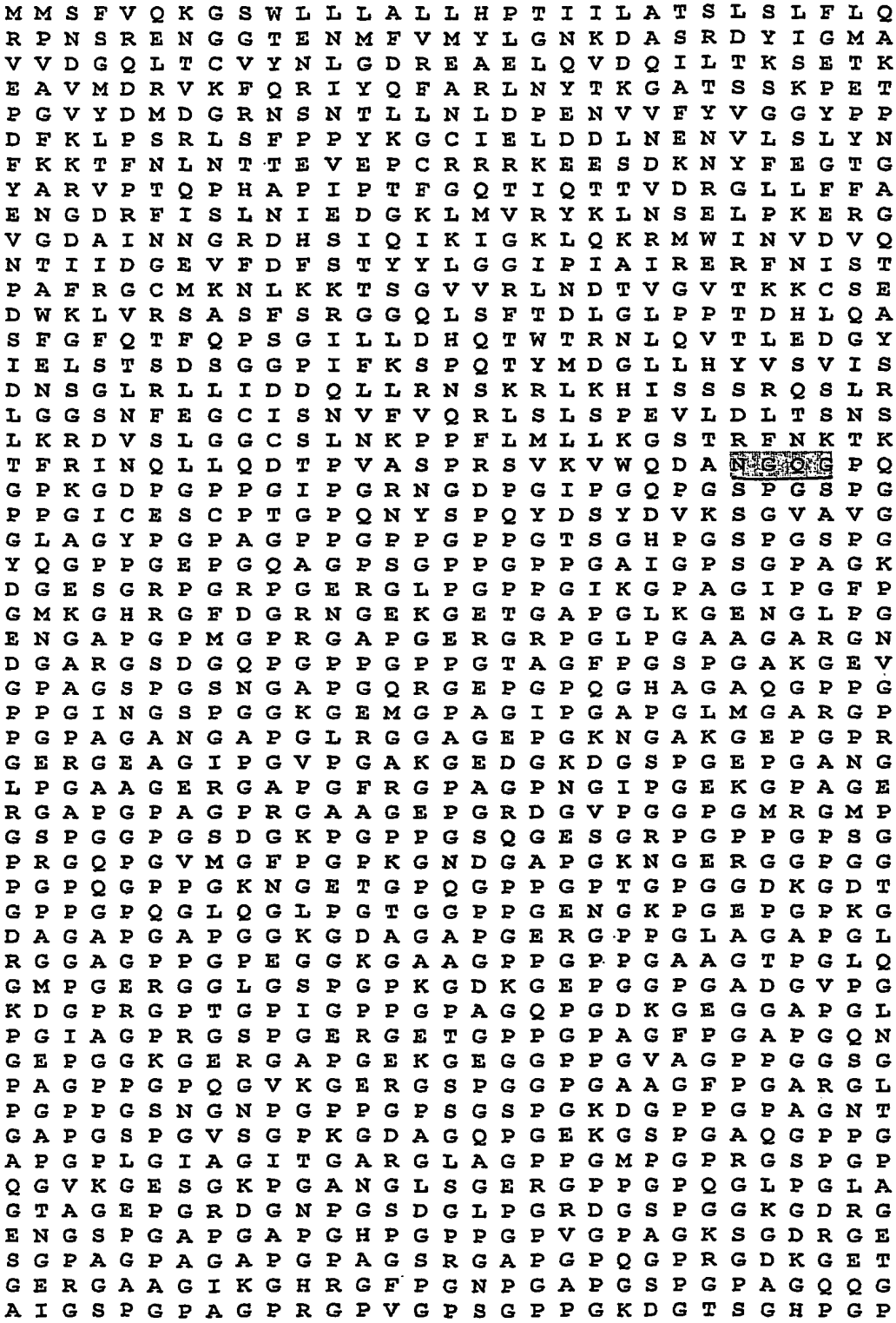
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Fig. 4 cont:

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Fig. 5

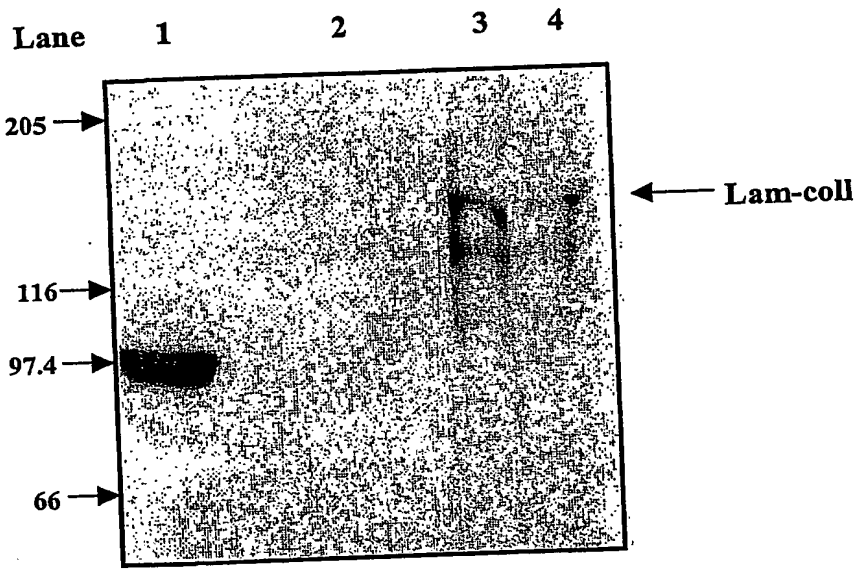

Fig. 6

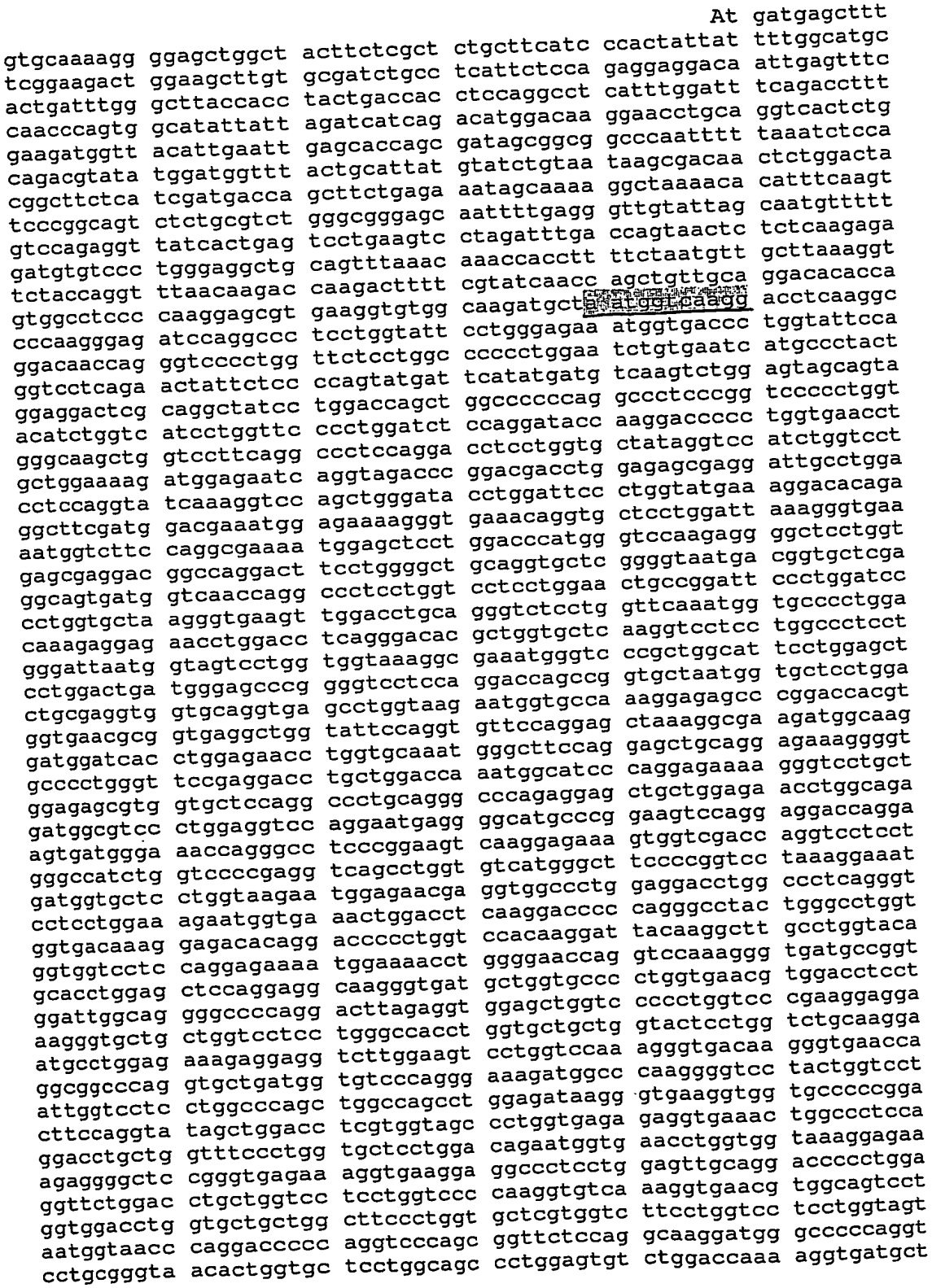
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Fig. 6 cont;

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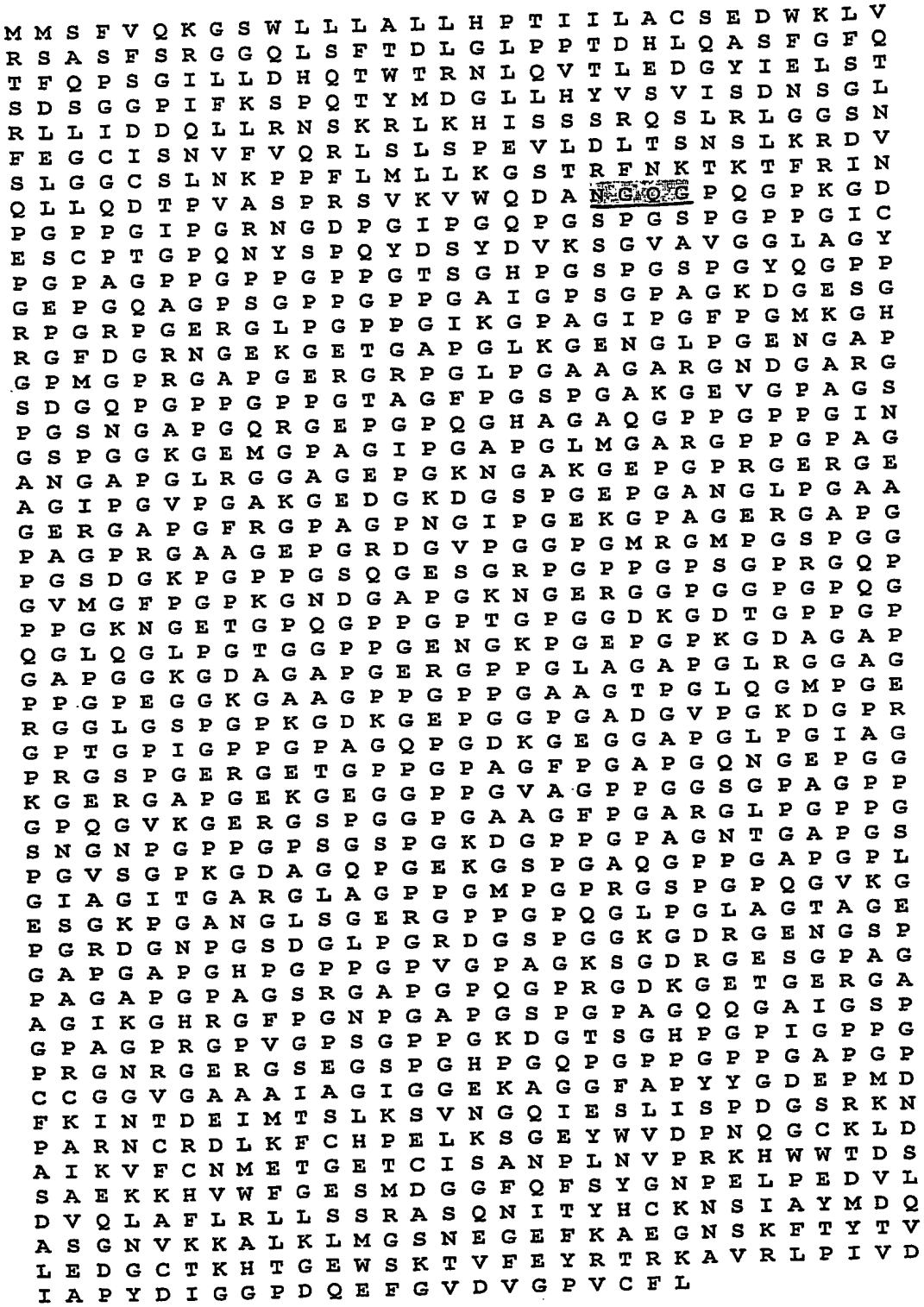
taa

Fig. 7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T F Q P S G I L L D H Q T W T R N L Q V T L E D G Y I E L S T
S D S G G P I F K S P Q T Y M D G L L H Y V S V I S D N S G L
R L L I D D Q L L R N S K R L K H I S S S R Q S L R L G G S N
F E G C I S N V F V Q R L S L S P E V L D L T S N S L K R D V
S L G G C S L N K P P F L M L L K G S T R F N K T K T F R I N
Q L L Q D T P V A S P R S V K V W Q D A N G G G P Q G P K G D
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G I A G I T G A R G L A G P P G M P G P R G S P G P Q G V K G
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Fig. 8

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Fig. 8 cont;

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Fig. 8 cont;

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Fig. 9

Met K S S G L F P F L V L L A L G T L A P W A V E G S G K S
F K A G V C P P K K S A Q C L R Y K K P E C Q S D W Q C P G
K K R C C P D T C G I K C L D P V D T P N P T R R K P G K C
P V T Y G Q C L Met L N P P N F C E Met D G Q C K R D L K C
C Met G Met C G K S C V S P V K A

A V E G G C S H L G Q S Y A D R D V W K P E P C Q I C V C D
S G S V L C D D I I C D D Q E L D C P N P E I P F G E C C A
V C P Q P P T A P T R P P N G Q G P Q G P K G D P G P P G I
P G R N G D P G I P G Q P G S P G S P G P P G I C E S C P T
G P Q N Y S P Q Y D S Y D V K S G V A V G G L A G Y P G P A
G P P G P P G P P G T S G H P G S P G S P G Y Q G P P G E P
G Q A G P S G P P G P P G A I G P S G P A G K D G E S G R P
G R P G E R G L P G P P G I K G P A G I P G F P G Met K G H
R G F D G R N G E K G E T G A P G L K G E N G L P G E N G A
P G P Met G P R G A P G E R G R P G L P G A A G A R G N D G
A R G S D G Q P G P P G P P G T A G F P G S P G A K G E V G
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P G A N G L P G A A G E R G A P G F R G P A G P N G I P G E
K G P A G E R G A P G P A G P R G A A G E P G R D G V P G G
P G Met R G Met P G S P G G P G S D G K P G P P G S Q G E S
G R P G P P G P S G P R G Q P G V Met G F P G P K G N D G A
P G K N G E R G G P G G P G P Q G P P G K N G E T G P Q G P
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P G E N G K P G E P G P K G D A G A P G A P G G K G D A G A
P G E R G P P G L A G A P G L R G G A G P P G P E G G K G A
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P K G D K G E P G G P G A D G V P G K D G P R G P T G P I G
P P G P A G Q P G D K G E G G A P G L P G I A G P R G S P G
E R G E T G P P G P A G F P G A P G Q N G E P G G K G E R G
A P G E K G E G G P P G V A G P P G G S G P A G P P G P Q G
V K G E R G S P G G P G A A G F P G A R G L P G P P G S N G
N P G P P G P S G S P G K D G P P G P A G N T G A P G S P G
V S G P K G D A G Q P G E K G S P G A Q G P P G A P G P L G
I A G I T G A R G L A G P P G Met P G P R G S P G P Q G V K
G E S G K P G A N G L S G E R G P P G P Q G L P G L A G T A
G E P G R D G N P G S D G L P G R D G S P G G K G D R G E N
G S P G A P G A P G H P G P P G P V G P A G K S G D R G E S

Fig. 9 cont;

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G P P G A P G P C C G G V G A A A I A G I G G E K A G G F A
P Y Y G D E P **Met** D F K I N T D E I **Met** T S L K S V N G Q I
E S L I S P D G S R K N P A R N C R D L K F C H P E L K S G
E Y W V D P N Q G C K L D A I K V F C N **Met** E T G E T C I S
A N P L N V P R K H W W T D S S A E K K H V W F G E S **Met** D
G G F Q F S Y G N P E L P E D V L D V Q L A F L R L L S S R
A S Q N I T Y H C K N S I A Y **Met** D Q A S G N V K K A L K L
Met G S N E G E F K A E G N S K F T Y T V L E D G C T K H T
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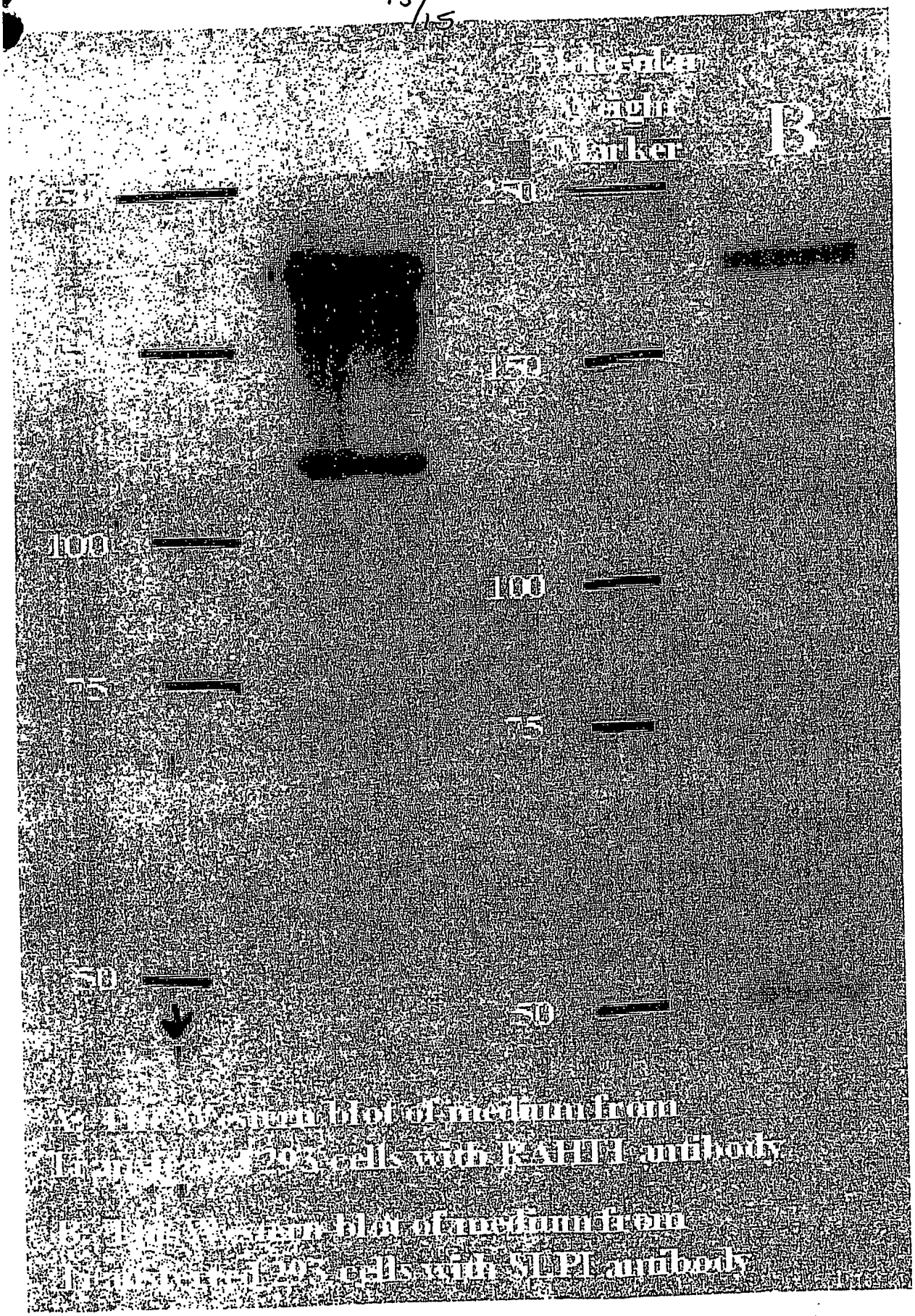

Fig. 10